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THE UNIVERSITY OF ALBERTA

# RESUSCITATION OF HEAT TREATED *ESCHERICHIA COLI*

by

Kanaan Khalifa (C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled RESUSCITATION OF HEAT TREATED ESCHERICHIA COLI submitted by Kanaan Khalifa in partial fulfillment of the requirements for the degree of Master of Science.

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Date October 16, 1975

ABSTRACT

The conditions and mechanism of heat injury and resuscitation were studies with two strains of Escherichia coli. The thermal death curves for these organisms were established at 52°C. For the heating period, strain SA211 gave nonlinear death curves on both plating media, while strain SA603 was linear on nutrient agar and nonlinear on the violet red bile agar plating medium, resulting in decreasing injury with extended heating.

Inhibitory levels of antibiotics were added to the resuscitation medium (tryptic soy broth), to determine their effect on resuscitation. The inhibition of resuscitation by chloramphenicol and tetracycline HCl, implicated protein synthesis in the repair mechanism. Resuscitation did not occur in the presence of amino acids, and the results confirmed that peptides were necessary in the resuscitation medium for the repair to occur.

Resuscitation occurred in media of limited nutrient capacity, such as 0.1% peptone water. A resuscitation medium that does not support growth, apart from those containing inhibitory levels of antibiotics, has not been discovered. From these studies it appears necessary either to improve the plating media or to develop a growth limiting resuscitation medium for use in routine methods of enumerating viable Escherichia coli in foods.

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TABLE OF CONTENT


Introduction ..... 1

Literature Review ..... 3

Materials and Methods ..... 16

Cultures ..... 16

Media ..... 16

Propagation ..... 16

Preparation and heat treatment ..... 17

Sampling and diluting ..... 18

Resuscitation ..... 18

Plating and incubation ..... 19

Antibiotic studies ..... 19

Resuscitation of *E. coli* on minimal media ..... 20

Calculations ..... 21

Replication ..... 21

Results and Discussion ..... 23

The effect of selective and nonselective media on the plate counts of unheated cells of *E. coli* ..... 23

Heat Injury of *E. coli* ..... 24

The resuscitation of injured *E. coli* ..... 27

The effect of antibiotics on the resuscitation of *E. coli* ..... 31

(i) Actinomycin D ..... 31

(ii) Ampicillin and vancomycin ..... 37

(iii) Chloramphenicol and tetracycline ..... 38

Effect of nutrients on the resuscitation of *E. coli* ..... 43

Conclusions ..... 51

References ..... 55

LIST OF TABLES


Table 1 Number of replications of trials for E. coli SA211 and SA603 ..... 22

Table 2 Optical density changes during resuscitation in tryptic soy broth at 37°C ..... 28

Table 3 Minimum inhibitory concentrations of antibiotics for the test organisms ..... 32

Table 4 Optical density changes during resuscitation of E. coli SA211 in tryptic soy broth, at 37°C, with and without actinomycin D ..... 36

Table 5 Resuscitation and growth response of E. coli SA603 in different resuscitation media ..... 45

Table 6 Resuscitation and growth response of E. coli SA603 in selected amino acids and other potential resuscitation media ..... 47

Table 7 The typical analysis of Bacto-peptone ..... 49

LIST OF FIGURES


Figure 1 Thermal death curves of E. coli SA211 plated on NA and VRBA ..... 25

Figure 2 Thermal death curves of E. coli SA603 plated on NA and VRBA ..... 26

Figure 3 Resuscitation curves for E. coli SA211 and SA603 plated on NA and VRBA ..... 30

Figure 4 Resuscitation curves for E. coli SA211 in tryptic soy broth in the presence of minimum inhibitory concentration of actinomycin D, ampicillin and vancomycin HCl ..... 33

Figure 5 Resuscitation curves for E. coli SA603 in tryptic soy broth in the presence of minimum inhibitory concentration of actinomycin D, ampicillin and vancomycin HCl ..... 35

Figure 6 Resuscitation curves for E. coli SA211 in tryptic soy broth in the presence of minimum inhibitory concentration of chloramphenicol and tetracycline HCl ..... 39

Figure 7 Resuscitation curves for E. coli SA603 in tryptic soy broth in the presence of minimum inhibitory concentration of chloramphenicol and tetracycline HCl ..... 40

Figure 8 Data presented by Roth (1969) for the resuscitation of heat treated E. coli SA211 with and without chloramphenicol ..... 41

# INTRODUCTION

During harvesting, processing, handling and distribution, foods may become contaminated with different microorganisms. Unsanitary handling, or contact with improperly cleaned equipment, or both, could result in the presence of coliform bacteria in a food. Public health authorities use coliform bacteria as an indicator of potential danger to health, while quality assurance laboratories use these bacteria as indicators of hygienic standards. Although differences exist in the interpretation of these tests by Public Health authorities and the Food Industry, there can be little doubt that these tests provide a guide to the standards of hygiene practiced in food handling.

More pertinent information to the hygienic standards of food handling can be obtained if the particular coliform bacteria contaminating the food are determined. Escherichia coli is a member of the coliform group of bacteria. This bacterium is commonly found in the intestinal tract of man and all vertebrates (Breed et al., 1957). The confirmed presence of E. coli type I in food would be more convincing evidence of fecal contamination, and a more significant indicator of a potential danger for human health. The detection of pathogens, or their indicator organisms, as well as the detection of spoilage organisms, is essential to a successful surveillance program for safe and economic

food supplies. However, there is increasing evidence to indicate that selective media currently in use often fail to detect all of the appropriate microorganisms.

The food microbiologist is concerned with the detection of microorganisms in foods. The value of this work is dependent upon the reliability of the tests used. Despite the evidence to indicate that environmental stresses in the processing of foods may cause a state of injury in the microorganisms, which increases their susceptibility to inhibitory agents in selective media, little has been done to take this into account in the standard methods for detecting microorganisms in foods. Microorganisms can resuscitate, and grow, to the detriment of quality assurance or safety for the consumer. Either improved selective media, which will support the growth of injured cells, or resuscitation procedures, which will allow injured cells to recover their ability to grow on selective media, must be developed.

The development of improved media or resuscitation procedures requires an understanding of cell injury and the resuscitation process. The object of this study was to determine the nature of injury and the requirements for resuscitation of sublethally heated strains of E. coli.

# LITERATURE REVIEW

In the late eighteenth century, Spallanzani noted lower heat resistance in "superior animalicula" (protozoa) than in "animalicula" (most probably bacteria) (Stanier et al., 1970). The first observation of heat sterilization has been attributed to Schwann (1837) who observed that if an infusion of meat was heated, and all of the air entering the enclosed flask was heated, putrefaction did not occur. In 1861 the application of heat treatment to foods was further advanced by Pasteur, and in 1876 Cohn discovered "spores" as a heat resistant form of bacteria (Stanier et al., 1970; and Wilson and Miles, 1957).

The first quantitative measurements of the heat resistance of organisms, to dry and moist heat, were made by Koch in 1881 and by Koch and Wolffhilgel in 1881. They found that the temperature required for the sterilization of spores with moist heat was lower than that required with dry heat (Smith et al., 1964; and Hugo, 1971). The possibility of sublethal heat treatment resulting in damage to the bacteria was introduced by Eijkman in 1908. She reported that the longer an organism was subjected to treatment with moist heat, the greater the period necessary for recovery (Harries, 1968).

The lethal action of moist heat on vegetative bacterial cells was studied as early as 1910, by Chick and Martin. Chick (1910) showed that the rate of death of a

population of vegetative bacteria was logarithmic. She stated that the disinfection of bacteria by heat in presence of water exhibits a striking analogy with the behavior of some proteins under similar conditions, and leads to the inference that disinfection of bacteria by heat is due to hydration of their constituents of proteins. This important work proposed a mechanism whereby moist heat killed bacteria and illustrated logarithmic death. This has been shown to be characteristic of a large number of other chemical and physical treatments lethal to microorganisms.

An increased lag phase of growth was observed after heat treatment. In 1923, Burke postulated that this was due to a heat resistant fraction of the culture. However, Hershey (1939) described a series of experiments in which the lag phase was increased with lengthened exposure to heat. He attributed the extended lag phase to injury of the cells, and not, as suggested by Burke (1923), selection of resistant cells that were inherently slow to develop. Extended lag phase as a response to thermal injury has been shown by many investigators with different organisms, e.g. Pseudomonas fluorescens (Lawton and Nelson, 1955), a Micrococcus strain (Kaufman et al., 1959), Staphylococcus aureus (Jackson and Woodbine, 1963) and Streptococcus faecalis (Clark et al., 1968).

The extended lag phase can be described as a period of recovery (repair, revival or resuscitation), during which the cell regains its ability to grow. In this study, "resuscitation" has been selected as the term to describe this process. Harris (1963) explained this as "the period of'getting back'

organisms from their sublethal environment". Roth (1969) defined "injury" as "the loss of some characteristic of the parent culture, which restricts the growth of the injured cells when this specific characteristic is challenged, but from which the injured cells are able to recover", and "recovery" (resuscitation) as "the repair of the damage responsible for injury, enabling previously injured cells to grow normally". This is commonly accomplished by incubating the organisms in a liquid medium such as tryptic soy broth (Roth, 1969).

Busta and Jezeski (1963) reported that, when staphylococci were subjected to a sublethal heat treatment, the survivor count on plate count agar was greater than on the selective medium, Staphylococcus medium No. 110. They demonstrated that the heat treatment made the cells salt sensitive, but that this salt tolerance returned if the treated cells were incubated in skim milk. This observation was also confirmed by Stiles and Witter (1965) and Iandolo and Ordal (1966), who further demonstrated that salt tolerance could be regained in a medium which would not support growth and multiplication of the organism. Strange and Shon (1964) noted a delay in the appearance of the colonies of Aerobacter aerogenes plated on solid media. They also noted a marked difference in colony size at certain incubation times, which indicated the possibility of a variable effect of the sublethal heat treatment on the cells.

Injury caused the organisms to be more exacting in

their nutritional requirements, to exhibit a narrower temperature and pH range for growth, to reduce their respiratory activity and oxygen consumption, and to increase their sensitivity to inhibitors and selective agents (Isaacs, 1930 a, b; Hershey 1939; Nelson, 1942; and Stiles and Witter 1965). Isaacs (1930b) suggested that organisms exposed to heat or chemicals might be damaged so that a medium which was optimal for growth without treatment, could be unsatisfactory for their growth after treatment.

Hershey (1939) found that the resuscitation of sublethally heat treated E. coli was better in liquid than on solid media. Nelson (1943) compared four agar media for their ability to support growth of damaged bacteria and found that beef infusion agar gave higher counts of heated bacteria than nutrient agar containing beef extract, and that both of these supported much higher counts than a simple synthetic medium. Nelson (1944) observed an increase in the number of heat treated bacterial cells growing on a synthetic medium, when tryptone had been added. Harris (1963) observed that yeast and malt extract added to beef extract broth increased the revival of phenol treated vegetative bacteria. Stapleton et al. (1955) found that the addition of yeast extract to minimal media gave increased resuscitation of irradiated bacteria, whereas Alper and Gillies (1958a,b) reported that for bacteria damaged by irradiation, resuscitation was better on minimal media, which were suboptimal for the growth of unheated cells. Kadota and co-workers in

1957, found that the addition of 0.1% yeast extract to a synthetic basal medium increased the plate counts of irradiated bacteria (Allwood and Russell, 1970). The same results were also obtained by Nakamura and Ramage (1965) on ultraviolet irradiated Shig. sonnei.

Addition of enriching agents does not necessarily mean that the growth rate will be greater. Harris (1963) reported that a simple meat extract medium was superior to a meat extract peptone medium for the recovery of phenol treated bacteria. Straka and Stokes (1959) observed that the addition of enrichments increased the revival of bacteria damaged by freezing. Nakamura and Dawson (1962), Roberts et al. (1965) and Postgate and Hunter (1963) obtained better recovery in nutrient agar than in a synthetic medium for freeze damaged Shig. sonnei, A. aerogenes and E. coli, respectively. Baird-Parker and Davenport (1965), on the other hand, reported very little difference between the media tested for the growth of frozen cells of S. aureus. Busta and Jezeski (1963) observed that heat shocked S. aureus exhibited sensitivity to sodium chloride in the growth medium. Stiles and Witter (1965) also observed this effect, and found that reducing or eliminating sodium chloride in the medium increased the plate counts. They also found that if the heat damaged cells were stored in 5% galactose or glucose before plating out, they regained their salt tolerance. Stiles and Witter (1965) and Iandolo and Ordal (1966) found no increase in the plate

counts on agars without salt during the resuscitation period, while plate counts on selective agars containing salt increased until they were similar to those on agars without salt.

Heinmets et al. (1954) observed that the addition of Krebs cycle metabolites to the medium "reactivated" E. coli damaged by heat and chemicals. Garvie (1955) reported the "multiplication" of damaged cells of E. coli in buffer solutions, plus metabolites. She considered the "reactivation" process observed by Heinmets and co-workers to be the result of the multiplication of a few survivors. Heather and Van der Zant (1957) found an increase in the number of viable cells during the incubation of heat damaged bacteria with isocitric acid, sodium citrate or a mixture of eleven metabolites. It would appear that all of these observations are in fact the occurrence of resuscitation of the injured microorganisms. Baird-Parker and Davenport (1965) reported that the addition of pyruvate to the growth medium increased the plate counts of S. aureus. This was not resuscitation in the same sense as the other observations. However, the presence of pyruvate in the growth medium is allowing the injured cells to grow, hence it must be assumed that resuscitation is occurring.

The sensitivity of bacteria to inhibitors greatly increased after heating. Jacobs and Harris (1961) reported that agars from different geographical sources gave different results when used in the cultivation of a number

of different bacterial species damaged by phenols. Jacobs and Harris (1960, 1961) and Harris (1963) found an increased "recovery" when media were detoxified. The presence of indicator dyes and selective agents in media also affected the plate counts of damaged bacteria. Roth (1969) found that sublethally heated E. coli showed an increased sensitivity to the bile salts used as the selective agent in many coliform media.

The optimum pH for recovery after heat treatment was not necessarily the optimum pH for growth of unheated organisms (Isaacs, 1930a,b). Nelson (1956) found that E. coli exhibited an optimum pH for recovery between 5.6-6.0, whereas unheated E. coli grew well over the pH range 5-9.

The temperature and the period of incubation affected the recovery of heated bacteria. Eijkman in 1908 reported that increasing the incubation period from 3 to 15 days, gave higher plate counts for the heated bacteria (Harries, 1968). Nelson (1942) reported that heat treated E. coli gave higher counts when incubated at 32°C than at 20 or 37°C. Nelson and Baker (1954) reported that pasteurized milk gave highest counts at 25°C and 21°C after 3 and 4 days' incubation, respectively. Roth (1969) found that resuscitation of heated E. coli was slower at 20°C than at 37°C, while there was no resuscitation at 4.4°C.

There are a number of possible mechanisms proposed to explain the lethal influence of moist heat on the vegetative cells of bacteria. These were listed by Strange

and Shon (1964) as follows:

1. Coagulation of protein.
2. Inactivation of enzymes.
3. Disruption of cellular lipids.
4. Damage to the genetic apparatus.
5. Breakdown of RNA.

Sykes (1962) suggested that moist heat coagulated bacterial proteins and denatured bacterial enzymes. Salton (1953) suggested that the susceptibility of the wall of the Gram-negative bacteria to rupture or damage on heating was due to the high lipid content or to the nature of the protein constituent. Harries and Russel (1966) found that lysozyme had no effect on unheated E. coli cells, nor on those which had been heated at 50°-60°C. This indicated either that lysozyme was unable to reach its mucopeptide substrate or that varying degrees of change in intracellular protein had prevented lysis. Lennox (1960) concluded that because boiled cells maintained their cell wall antigenicity, the cell walls of Gram-negative bacteria were not affected by high temperatures. The cell wall of staphylococci was not affected by heat treatment up to 100°C (Salton, 1953), and this was confirmed in studies involving electron micrographs of ultra thin sections of cells from a heated suspension of S. aureus (Allwood and Russell, 1969).

It was found that heating caused the leakage of cellular constituents, including RNA-like material. Califano in 1952 reported that the amount of nucleic acid

in the supernatant of a washed suspension increased with time and temperature of heating (Allwood and Russell, 1970), while Harries (1968) reported that Cantelmo in 1950 had found that the amount of nucleic acid liberated increased as the age of culture increased. Mitchell (1951) suggested that the osmotic barrier of bacterial cells might be damaged by heat treatment. There appeared to be a link between loss of viability and the leakage of small molecules from the cell which occurred, at least partially, as a result of damage to the cytoplasmic membrane. Pethica (1958) considered such leakage a manifestation of membrane damage. Strange and Shon (1964) showed that a close relationship existed between leakage of RNA-like material from cells and death of A. aerogenes stored at 47°C. The influence of temperature on the physiochemical structure of the lipid membranes was studied by Byrne and Chapman (1964). It was suggested that the cytoplasmic membrane would be expected to be very sensitive to temperature changes since such membranes existed on the borderline of a "temperature-sensitive phase transition".

Wood (1956) suggested that nuclear damage could be the result of high temperature inactivation of bacteria and other unicellular organisms. Szybalski (1967) found that the denaturation of the native, double-stranded DNA is, presumably, a collapse of the hydrogen-bonded, double-helical structure, leading eventually to a dissociation of the complementary strands. He found an increase in extinction

of DNA at 260 nm and a decrease in its viscosity. However, DNA must be heated to high temperatures before its structure collapses, and the cleavage of hydrogen bonds at high temperatures is often a gradual process.

The term "denaturation" is used here to denote the disruption of an original, natural, largely hydrogen-bonded and complex structure. Presumably, heat induces a breakage of many of the hydrogen bonds and an unfolding of the polypeptide chain with a collapse of the native protein structure (Allwood and Russell, 1970). In general terms, protein denaturation might be completely reversible under some carefully controlled conditions, dependent on the degree to which denaturation has occurred. It is questionable, therefore, whether bacteria possess a mechanism which enables them to "repair" this limited denaturation. However, the basis for the revival of heat damaged cells on nutritionally complex media did not indicate that protein renaturation was involved. Moreover, loss of viability of heated bacteria appeared before protein coagulation could be detected. This intracellular protein coagulation occurred above a particular temperature of exposure, but it was shown to be the primary cause of thermally induced death of vegetative bacteria (Allwood and Russell, 1970).

Few experiments have been carried out with a view to making an assessment of the role of enzyme inactivation in bacterial death. One study by Rahn and Schroeder (1941) showed that whereas there was 99% kill of Bacillus cereus

after ten minutes at 46°C, the reduction in peroxidase and catalase activity was only 14 and 20%, respectively. The heat inactivation of formic dehydrogenase in whole cells of a facultatively psychrophilic strain of E. coli was far greater than in the mesophilic strain under the same conditions (Upadhyay and Stokes, 1963). Evison and Rose (1965) suggested that the death of psychrophilic bacteria above their maximum growth temperature could be due to heat inactivation of their constituent enzymes. Allwood and Russell (1970) reported that in nonsporing organisms, some enzymes were more thermolabile than others, and consequently, that the site of an enzyme in the cell might play a part in determining its inactivation by heat.

As early as 1935, Belehradek stated that the effects of high temperature upon living systems were so complex that no theory could be ventured to reduce them all to a uniform basis. Studies to find the mechanisms of heat injury have taken a variety of routes.

Antibiotics have been used as research tools to elucidate the mechanisms involved. When chloramphenicol was added to growing cells at concentrations of 10 µg per ml and above, 95 to 100% of protein synthesis was inhibited. This was shown for S. aureus by Gale and Folkes (1953) and for E. coli by Wisseman et al. (1954). Roth (1969) found that the presence of chloramphenicol in the resuscitation medium inhibited the recovery of the heat induced sensitivity to violet red bile agar. Mukherjee and

Bhattacharjee (1970) observed that when E. coli was grown in the presence of chloramphenicol (100 µg/ml) for 30 minutes before heating, the resuscitation process was stopped. The resuscitation phenomenon was re-established when the organisms were subsequently allowed to grow in the absence of chloramphenicol for a period of at least 30 minutes before exposure to heat. They concluded that there was a direct involvement of protein in the mechanism of heat injury.

Inhibition of protein synthesis by tetracyclines was observed by Gale and Folkes (1953) who pointed out that their action was similar to that of chloramphenicol. Park (1959) and Hash (1963) reported that tetracyclines did not inhibit cell wall synthesis. Franklin (1963) showed that a number of tetracyclines strongly inhibited the transfer of amino acids from aminoacyl-sRNA to ribosome-bound protein, but had no effect on the binding of sRNA to ribosomes. He suggested that tetracyclines might specifically inhibit the formation of peptide bonds.

Jordan (1961) found that vancomycin rapidly inhibited the incorporation of amino acids into the cell wall fraction of S. aureus, while protein synthesis continued for some time. It also caused the accumulation of uridine nucleotides in the cells. The activity of ampicillin was four to eight times greater than that of benzyl penicillin against various Gram-negative bacilli and most strains of E. coli. Ampicillin inhibited

the synthesis of peptidoglycan and D-alanine carboxypeptidase in E. coli (Gottlieb and Shaw, 1967).

The first indication that actinomycin D was an inhibitor of nucleic acid synthesis came from the work of Kirk (1960) and Slotnick (1960), who found that RNA and protein synthesis in growing bacteria were inhibited by this antibiotic, but that DNA synthesis was much less sensitive to it. The actinomycins have been extremely useful in investigations concerned with DNA synthesis, RNA, and virus multiplication (Davis and Feingold, 1962). Heat injured Strep. faecalis cells were observed to recover their tolerance to sodium chloride, sodium azide, and bromocresol purple when incubated in tryptic soy broth (Clark, Witter and Ordal, 1968). Actinomycin D inhibited this recovery process and thus demonstrated the requirement for RNA synthesis in the recovery mechanism of Strep. faecalis.

Considerable work has been carried out to show that resuscitation of heat treated bacteria is affected by the medium used subsequent to the sublethal heat treatment. Little work has been done to find the exact mechanisms of thermal injury, and the requirements for heat treated cells to resuscitate.

# MATERIALS AND METHODS

## Cultures

Streptomycin resistant strains of E. coli, SA211 (E. coli B) and SA603 (E. coli K12) were used in this study. They were originally obtained from Dr. K. Sanderson, Department of Biology, University of Calgary, Calgary, Alberta. A freeze dried culture of these strains was prepared and stored for future use in case of changes in the original strains.

## Media

Tryptic soy broth (TSB) was used as the growth medium for maintaining the cultures and for the production of cells for heat treatment. Nutrient agar (NA) was used as the control medium and violet red bile agar (VRBA) was the selective medium. All media were obtained in the dehydrated form from Difco and rehydrated as directed. NA that was not used immediately was stored in a refrigerator at 40°F until needed, at which time it was melted in flowing steam for 20 to 25 min.

## Propagation

The organisms were transferred daily into 100 ml sterile tryptic soy broth (Difco) in a 250-ml pyrex Erlenmeyer flask and incubated at 37°C for 18 to 24 hr under constant aeration in a New Brunswick controlled Environment Incubator Shaker, Gyrotory Model G25, shaking at 200 r.p.m. with an

eccentricity of 1 inch. Periodic examination of a Gram-stained smear was done to ensure the purity of the cultures. Periodic testing of the organisms for their streptomycin resistance was made as follows: streptomycin sulfate powder (Pfizer) was dissolved in distilled water, and filter sterilized (Metricel autoclavable membrane filter, 0.20 μ pore size). The streptomycin solution was added aseptically to sterile nutrient agar at 46°C to give a final concentration of 200 μg/ml. Plates were poured and 0.1 ml aliquots of the test organisms were surface streaked in triplicate, with a sterile glass "hockey stick". The plates were incubated at 37°C for 30 hr, and colonies examined for streptomycin resistance.

Preparation and Heat Treatment

A three ml aliquot of the actively growing stock culture was inoculated into 100 ml sterile TSB in a 250-ml pyrex Erlenmeyer flask, to give an initial $OD_{600}$nm of approximately 0.1, and incubated at 37°C under constant aeration as indicated for culture propagation, for 3-3 1/2 hr, until the $OD_{600}$nm had increased to 1.0 ± 0.02. The culture was harvested by centrifugation in a refrigerated centrifuge (Sorvall RC-2B, GSA head) at 5000 r.p.m., for 5 min at 0 to 2°C. The supernatant was decanted and the pellet of cells resuspended in 2 ml sterile, 0.85% (w/v) saline solution.

A one ml aliquot of the resuspended cells was transferred to 99 ml preheated TSB in a 250-ml pyrex Erlenmeyer flask. The flask was suspended in a circulating water

bath, supplied with constant temperature water from a Colora, recirculating water bath at 52°C (unless otherwise specified). The broth was stirred by means of a 1 1/2 inch Teflon-covered stirring bar over a magnetic stirrer. The speed of the stirrer was adjusted to obtain maximum agitation without losing the rotational, stirring effect. The heating system was a modification of that used by El-Bisi and Ordal (1956), and used by Roth (1969).

Sampling and Diluting

The first sample was withdrawn 30 sec after inoculation. Successive 1 ml aliquots of the heated cell suspension were removed, by pipet, at specified time intervals during the heating and resuscitation periods. The samples were transferred into 99 ml precooled (0°C, in an ice bath), sterile 0.1% peptone water blank, for further dilution and plating. The dilutions were handled according to standard methods (Thatcher and Clark, 1968). In some cases, exceptions to this procedure were necessary, for example, where the expected concentration of the viable organisms/ml was such that 0.1 or 1.0 ml aliquots of the heated cells had to be plated.

Resuscitation

After the heating period, the reaction flask was cooled to 37°C in an ice bath, and incubated at 37°C for resuscitation. Samples were withdrawn at selected intervals and used to determine the optical density. These samples

were also diluted in 99 ml, cooled, 0.1% peptone water blanks and plated to determine their viable counts. Sampling was continued either until an increase in the optical density was observed, indicating that growth of the organisms had commenced, or for 6-8 hr, to include part of the growth phase of the cells.

Plating and Incubation

After dilution, and without delay, 0.1 ml or 1.0 ml of suitable dilutions were inoculated in triplicate into appropriately marked plastic petri dishes (15 X 100 mm). Approximately 15 ml of the sterilized media, NA and VRBA, previously cooled to 46°C were poured into the appropriate petri dishes. The agar was mixed with the inoculum by rotating the dishes 10 times clockwise and 10 times counter-clockwise (Hall, 1970). The medium was allowed to solidify, and the VRBA plates were covered with 4 to 5 ml overlay of the same medium, as recommended (Difco). The plates were then inverted and incubated at 37°C. The VRBA plates were incubated for 18 to 24 hr and the nutrient agar plates for 44 to 48 hr before counting. The colonies were counted using a New Brunswick Electronic Colony Counter (Model C-110).

Antibiotic Studies

Antibiotics were selected to establish the possible type of heat damage to the cells. The antibiotics were added to the resuscitation media (TSB) in the following concentrations:

1. Actinomycin D (Merck, Sharp and Dohme) 75 and 100 µg/ml
2. Ampicillin (Ayerst Laboratories) 2 µg/ml
3. Chloramphenicol (Park, Davis and Company) 2.5 µg/ml
4. Tetracycline (Novopharm Ltd.) 2 µg/ml
5. Vancomycin (Eli Lilly and Co. (Canada) Ltd.) 100 µg/ml

All other conditions of the experiment were carried out as described in the foregoing procedures. Controls without antibiotics were carried out with each antibiotic experiment.

Resuscitation of E. coli in minimal media

The test organism used in this study was E. coli SA603. An inoculum of these cells from a 24 hr stock culture was grown in TSB as described in propagation (p. 16) and preparation and heat treatment (p. 17). The cells were harvested by centrifugation (5000 r.p.m., Sorvall RC-2B, GSA head, 0-2°C), the supernatant was discarded, and the cells washed twice with sterilized, distilled water. A 2 ml aliquot of washed, resuspended cells was inoculated into specified media.

Growth was followed by measuring the optical density at 600 nm and the viable count was determined by plating appropriate dilutions of samples on NA and VRBA. Samples were taken after inoculation and at two-hour intervals. The plates were incubated at 37°C, as specified under plating and incubation (p. 19).

## Calculations

A computer program was written for the IBM 360/67 to calculate and plot the logarithm of the number of organisms against time. The program accepts the data as the number of organisms at various times and calculates and plots the best fitting curve.

## Replications

The number of replications for each trial is shown in Table 1.

Table 1. Number of replications of trials for E. coli SA211 and SA603.

| Experiments | Number of Trials SA211 | SA603 |
|---|---|---|
| Heating injury | 4 | 6 |
| Heating and resuscitation in TSB | 12 | 5 |
| Resuscitation with 2.5 µg/ml chloramphenicol | 2 | 2 |
| Resuscitation with 100.0 µg/ml actinomycin D | 3 | 2 |
| Resuscitation with 2.0 µg/ml tetracycline HCl | 2 | 2 |
| Resuscitation with 2.0 µg/ml ampicillin | 2 | 2 |
| Resuscitation with 100.0 µg/ml vancomycin HCl | 2 | 2 |
| Resuscitation in minimal media | nil | duplicate trials |

## RESULTS AND DISCUSSION

### The effect of selective and nonselective media on the plate counts of unheated cells of E. coli.

Viable counts were determined on unheated E. coli cells. Appropriate dilutions were plated onto selective violet red bile agar (VRBA) and nonselective nutrient agar (NA) media. The plate counts were compared after incubation at 37°C for 18 to 24 hr and 44 to 48 hr, respectively. After 24 hr incubation, the colonies on NA were visible but too small to count accurately. The percent difference in the mean counts for the two strains were: E. coli SA211, NA 33.6% higher than VRBA, and E. coli SA603, NA 25.1% higher than VRBA. These differences confirmed the observations by Roth (1969), but in 1971, Roth and Keenan reported no difference in the viable counts for these two strains on NA and VRBA.

The unheated strains, grown in stock culture were observed to change with continuing daily subculturing. The most noticeable changes were:-

(i) The appearance of small colonies on VRBA that did not precipitate the bile salts.

(ii) these small colonies were difficult to count after 24 hr incubation, and required extended incubation for 12 hr similar to the requirements on NA.

(iii) the heat resistance increased, especially with strain SA603, but the extent of injury observed on VRBA decreased.

To avoid errors from these changes in the cultures, as soon as these changes were observed, a new freeze dried culture of the original bile salts precipitating strain was cultured. This new culture was subcultured on three successive days in tryptic soy broth, before use in the heating experiments.

Heat Injury of E. coli

The two strains of E. coli, SA211 and SA603 were used in this preliminary experiment to determine the effect of time and temperature of heating on the death rate of these strains. The cells were heated for an extended period of time, and the viable counts were determined in NA and VRBA. The results are shown in Figures 1 and 2. The differences between the counts obtained on VRBA and NA were greater than the differences for the unheated cells. This increased difference in the counts was considered to be "heat injury". This was defined by Roth (1969) as "The loss of some characteristic of the parent culture, which restricts the growth of the injured cells when this specific characteristic is challenged, but from which the injured cells were able to recover."

From the data (Fig. 1 and 2), it can be seen that the percent injury reached a maximum after 20 min of heating. Roth's (1969) observations of heat injury were carried out for 15 min. The data for this study were obtained over a 1 hr heating period. Trials on E. coli SA603 were also extended to 3 hr. After 20 min, the rate of injury decreased, as shown by the nonlinearity of the death curves for both strains on VRBA. The death curve

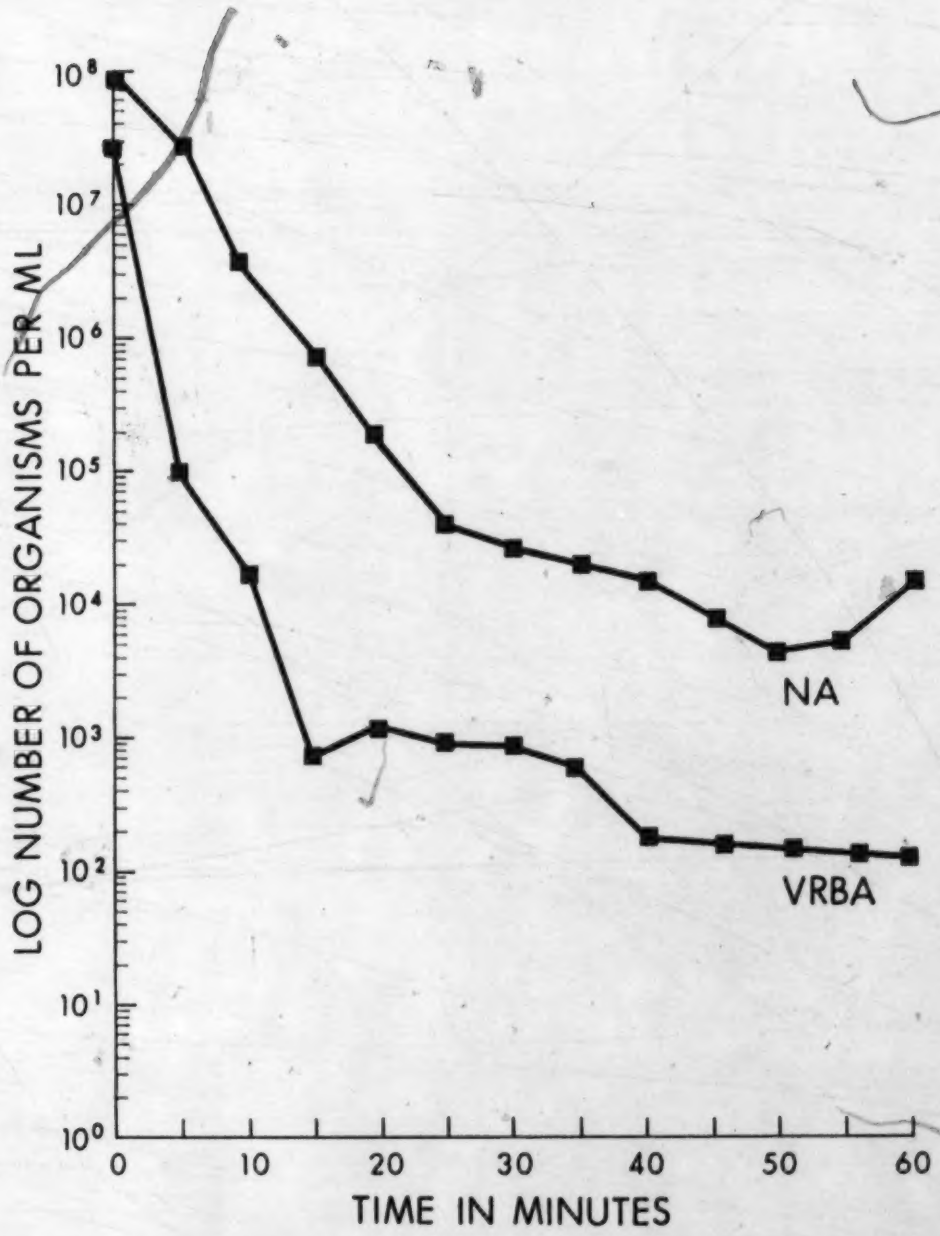


Figure 1. Thermal death curves of E. coli SA211 plated on NA and VRBA.

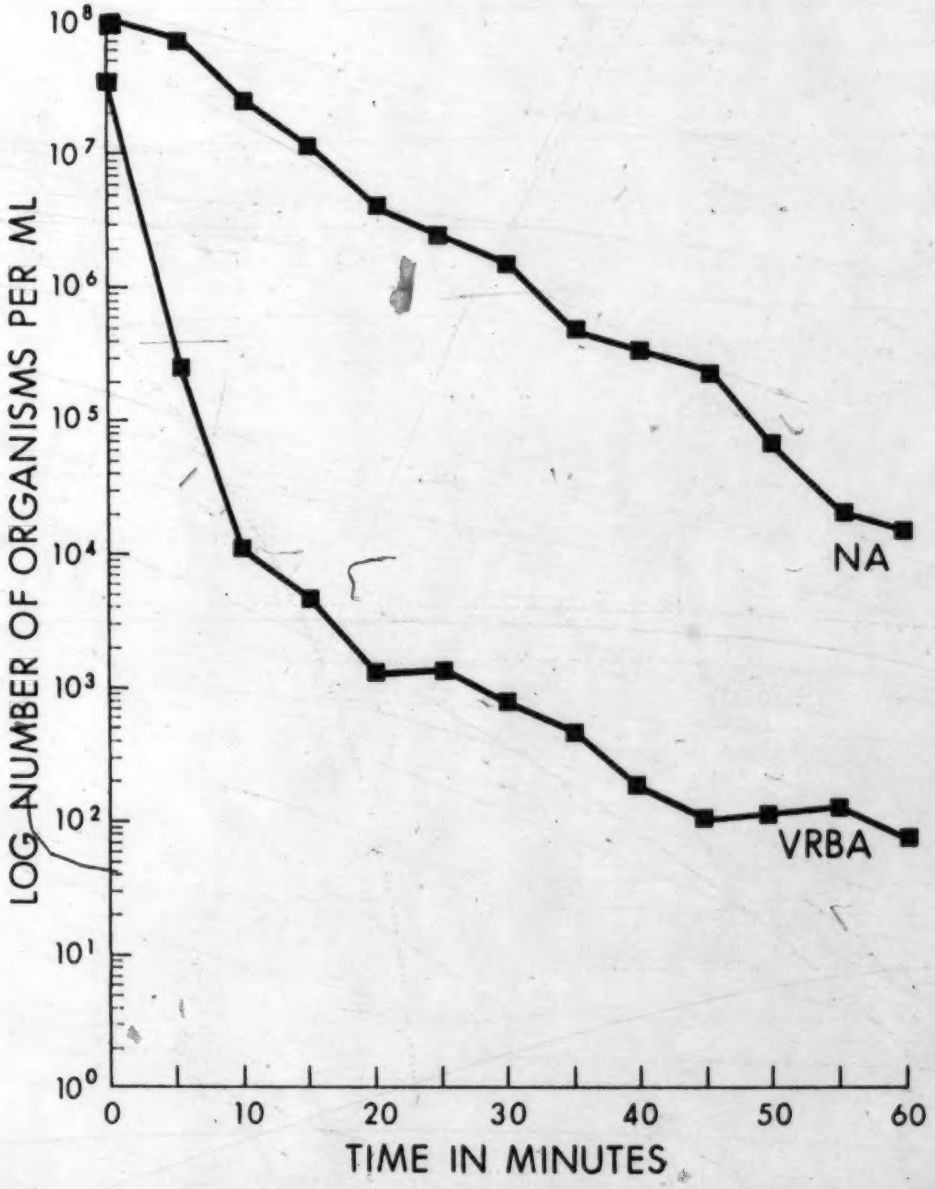


Figure 2. Thermal death curves of E. coli SA603 plated on NA and VRBA.

for strain SA211 on NA, was nonlinear, giving an almost constant level of injury (95-99%) after 20 min heating. For strain SA603, however, the death curve on NA was still linear after 60 min of heating at 52°C, with the effect that decreasing levels of injury were being observed with extended time of heating. As a result three trials on SA603 were extended to 150, 160, and 180 min. After 60 min heating, the death curve for SA603 became nonlinear and a constant level of injury resulted.

These data confirmed that extended death curves of E. coli were nonlinear. Although the reliability of the selective medium was better with extended periods of heating, the counts were still lower by a 10 to 100 fold factor. Similarly, the initial, frequently linear death curves could not be extrapolated to predict death rates with extended heating.

## The resuscitation of injured E. coli

These experiments were conducted to confirm the observation of Roth (1969) that these experimental strains of E. coli, after heat damage, could resuscitate and grow in a favorable resuscitation medium. Representative results for these death and resuscitation curves were obtained from a computer program designed to give the best fit for the data. The curves were obtained from 12 trials with strain SA211 and 5 trials with strain SA603. During the resuscitation period, the optical density of the resuscitation broth was determined at each sampling time. The data in Table 2 indicated that after heating

Table 2. Optical density changes during resuscitation in tryptic soy broth at 37°C

| TIME (hr) | E. coli SA211 $OD_{600\ nm}$ | | E. coli SA603 $OD_{600\ nm}$ | |
|---|---|---|---|---|
| 0 | 0.40 | | 0.56 | |
| ½ | 0.39 | | 0.555 | |
| 1 | 0.38 | | 0.55 | |
| 1½ | 0.38 | | 0.54 | |
| 2 | 0.37 | | 0.535 | |
| 2½ | 0.37 | | 0.535 | |
| 3 | 0.37 | | 0.535 | |
| 3½ | 0.37 | | 0.535 | |
| 4 | 0.37 | | 0.535 | Lag |
| 4½ | 0.39 | | 0.55 | Growth |
| 5 | 0.38 | Lag | 0.56 | |
| 5½ | 0.40 | Growth | 0.62 | |
| 6 | 0.45 | | 0.67 | |

the lag phase extended for 5 hr (SA211) and 4 hr (SA603).

The resuscitation data are shown in Figure 3. The resuscitation rates varied. On NA, there was a marked resuscitation during the first 30 min of incubation. Thereafter both strains continued to show increases in numbers until approximately 1½ to 2 hr prior to growth. Roth (1969) did not refer to this continuing resuscitation in his data for NA. However, the data from both studies indicated that, despite the increase in numbers on NA, the increase could be attributed to resuscitation, and not growth. At the same time, resuscitation occurred on VRBA. The initial rate was rapid, but decreased with time, until the injured cells were fully resuscitated, prior to growth commencing. For strain SA211 there was a marked decrease in resuscitation rate after 1 hr. Resusciation re-commenced after 2 hr prior to growth, the differences between the counts obtained on NA and VRBA were similar to the differences observed for unheated cells.

Harries (1968) studied the effect of adding yeast extract (1% w/v) to NA, on the growth of heat damaged E. coli cells. She observed that the counts first decreased, then increased, so that after 4 hr incubation, the count had almost reached the count observed before heating. The data for this study indicated a similar result (Fig. 3). Like Roth (1969), Harries also suggested that the cells were damaged with respect to their ability to grow on NA.

The exact damage in sublethally heated cells is not known. The terms revival, repair and recovery have been used to describe this increase in the number of cells capable of growing

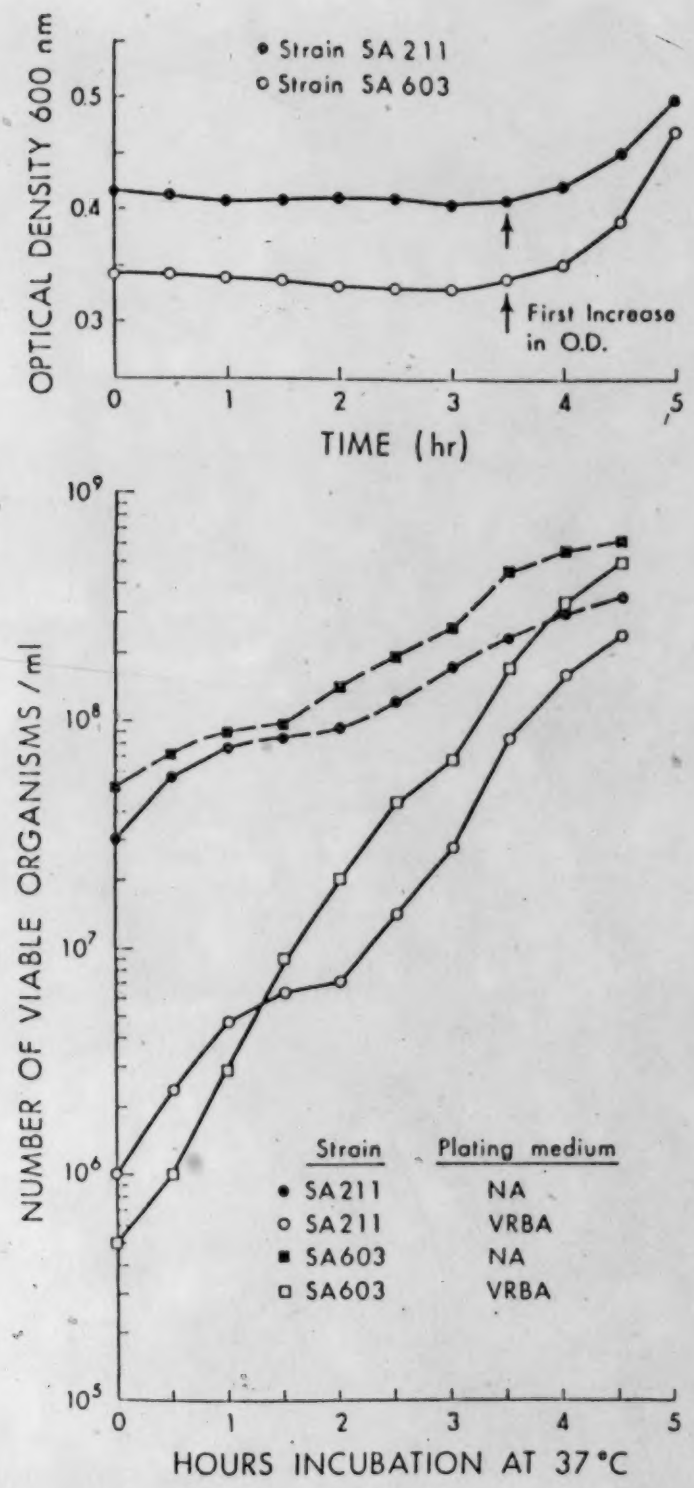


Figure 3. Resuscitation curves for E. coli SA211 and SA603 plated on NA and VRBA.

on plating media, during post-heating lag. The change involved could be resynthesis of enzymes, RNA, or the damaged part of the cells. The term resuscitation, meaning a "revival from apparent death" (Funk and Wagnalls, 1963) was considered to be the appropriate term for use in this study.

## The effect of antibiotics on the resuscitation of E. coli

The concentration of antibiotics required for use in the resuscitation media was determined from the minimum inhibitory concentration data for each antibiotic with both strains of E. coli. The results are given in Table 3. The experiments in this section were designed to extend the observation by Roth (1969), that chloramphenicol at 2.5 µg/ml in the resuscitation medium inhibited the resuscitation of the injured cells on VRBA. Mukherjee and Bhattacharjee (1970) reported a related observation for E. coli B "growing" in the presence of 100 µg/ml chloramphenicol before heat treatment. The injured cells failed to resuscitate after heat treatment at 52°C.

The results for the resuscitation of the test strains of E. coli, in the presence of the specified concentrations of antibiotics, are presented in Figures 4, 5, 6 and 7.

(i) Actinomycin D

E. coli SA211, in the presence of actinomycin D, 100 µg/ml, in the resuscitation broth (Fig. 4), indicated that resuscitation occurred for both NA and VRBA in the presence of this antibiotic. At the time that growth commenced, (approximately 3 hr, see Table 4) resuscitation was incomplete. The difference between NA and VRBA counts decreased

Table 3. Minimum inhibitory concentrations of antibiotics for the test organisms.

| | Antibiotic | E. coli SA211 (µg/ml) | E. coli SA603 (µg/ml) |
|---|---|---|---|
| 1. | Actinomycin D | 100.0 | 75.0 |
| 2. | Ampicillin | 2.0 | 2.0 |
| 3. | Vancomycin HCl | 100.0 | 100.0 |
| 4. | Chloramphenicol | 2.5 | 2.5 |
| 5. | Tetracycline HCl | 2.0 | 2.0 |

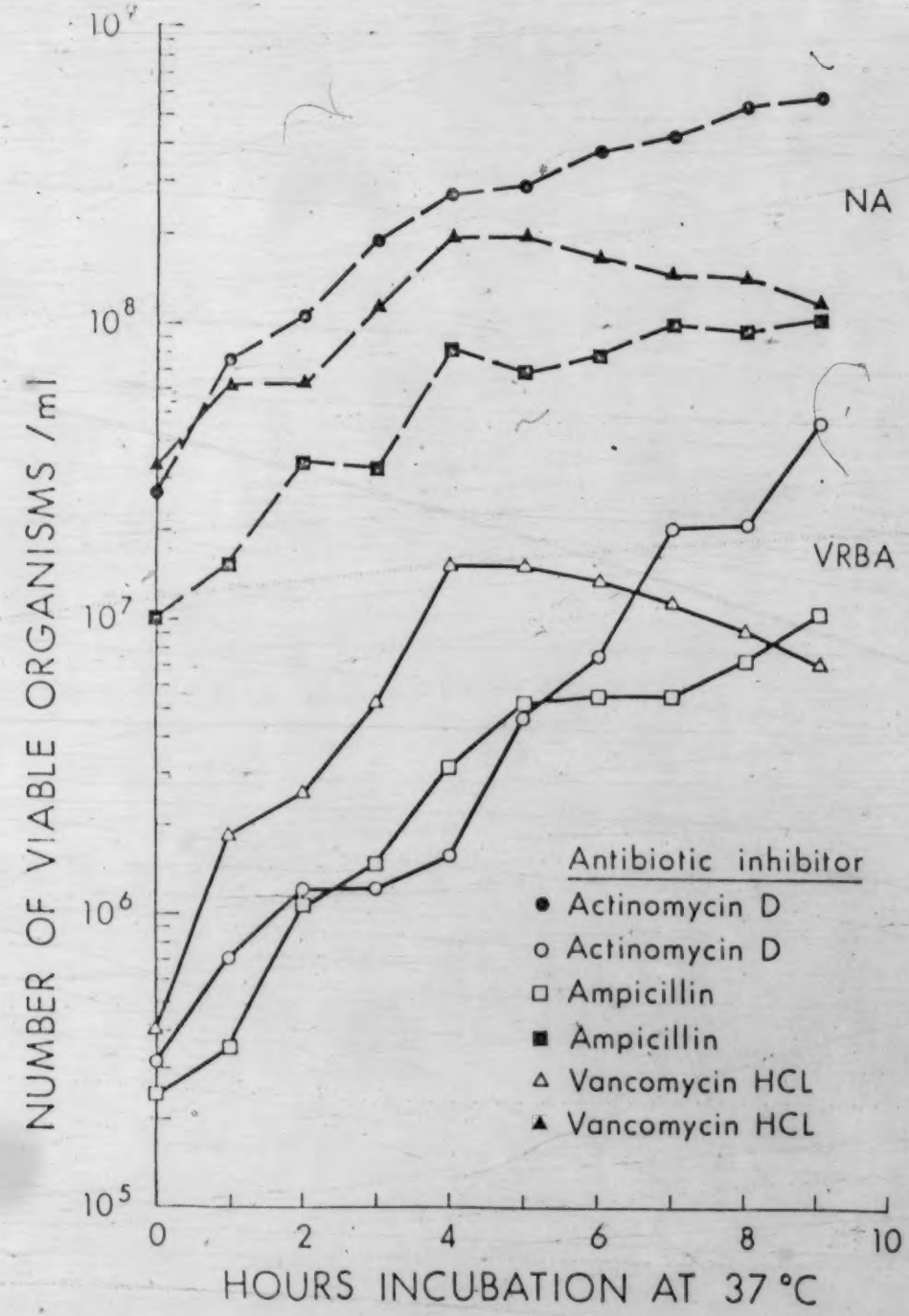


Figure 4. Resuscitation curves for E. coli SA211 in tryptic soy broth in the presence of minimum inhibitory concentration of actinomycin D, ampicillin and vancomycin HCl.

as growth progressed. The data suggest that Actinomycin D at the previously determined minimum inhibitory concentration of 100 µg/ml in the resuscitation broth does not inhibit growth. However, this might be due to the permeability problem with this antibiotic, hence Actinomycin D cannot readily be used to determine the mechanism of resuscitation.

For the strain SA603 (Fig. 5), with actinomycin D added to the resuscitation broth, there appeared to be a gradual increase in the number of colonies growing on NA during the growth period. Since the OD indicated that numbers were increasing for both SA211 and SA603 in the presence of actinomycin D, it was difficult to state that growth did not occur in the presence of this antibiotic. Kirk (1960) noted that the extent of inhibition obtained at a given concentration depended on the density of the cell suspension.

Actinomycin D has a very specific action and was reported to form complexes with DNA (Kirk 1960). It was reported (Reich, Cerami and Ward, 1967) that actinomycin D forms complexes with DNA, but not with other cellular constituents. The addition of actinomycin D to an exponentially growing culture of S. aureus produced immediate inhibition of RNA synthesis, followed rapidly by inhibition of protein synthesis. Inhibition of DNA synthesis occurred after a 10-15 min lag. The degree of DNA inhibition for a given concentration of actinomycin D was less than that occurring for RNA or protein synthesis. Iandolo and Ordal (1966) found that

Figure 5. Resuscitation curves for E. coli SA603 in tryptic soy broth in the presence of minimum inhibitory concentration of actinomycin D, ampicillin and vancomycin HCl.

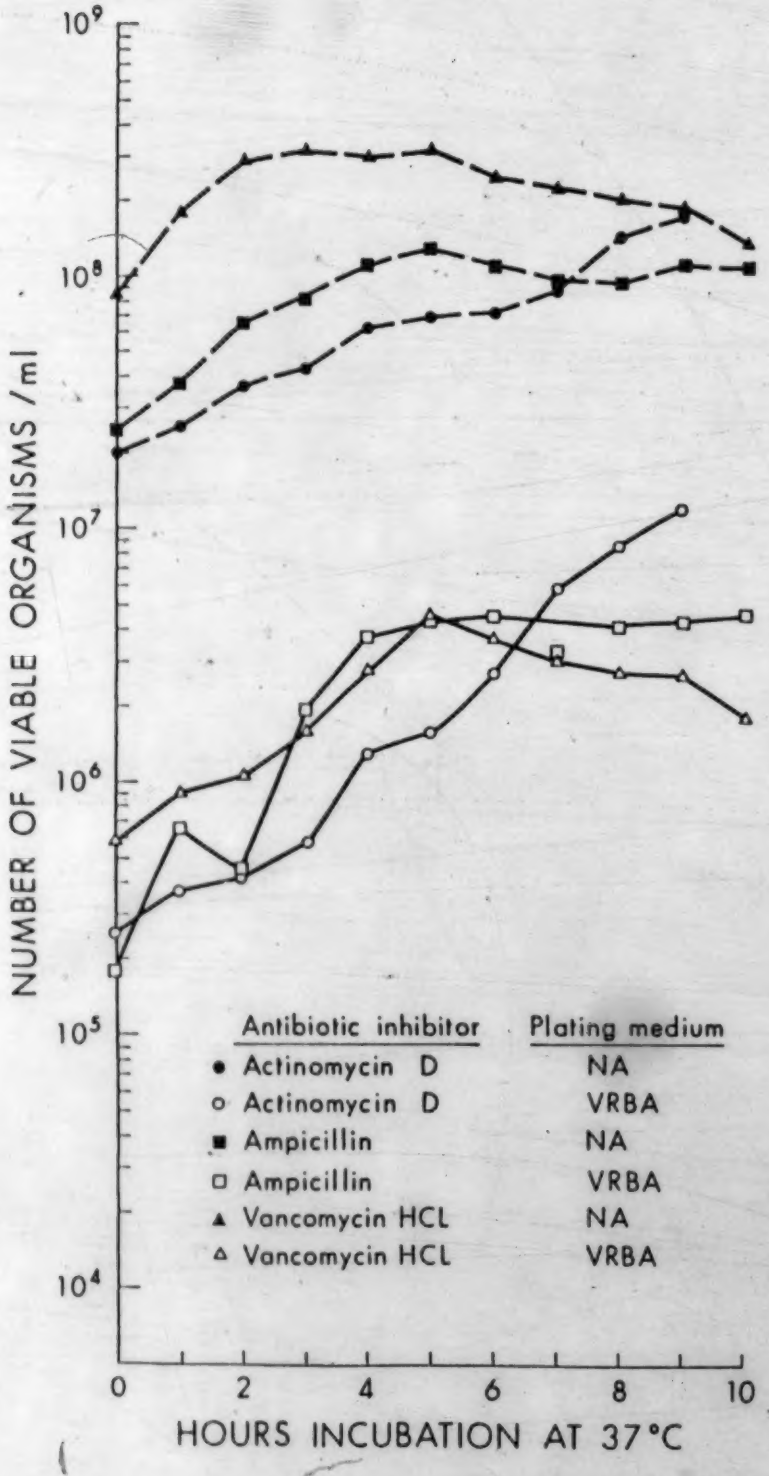
NUMBER OF VIABLE ORGANISMS / ml
$10^9$
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
Antibiotic inhibitor
Plating medium
Actinomycin D
NA
Actinomycin D
VRBA
Ampicillin
NA
Ampicillin
VRBA
Vancomycin HCL
NA
Vancomycin HCL
VRBA
0
2
4
6
8
10
HOURS INCUBATION AT 37 °C

Table 4. Optical density changes during resuscitation of *E. coli* SA211 in tryptic soy broth, at 37°C, with and without actinomycin D.

| TIME (hr) | With Actinomycin D (100 µg/ml) ($OD_{600nm}$) | Without Actinomycin D ($OD_{600nm}$) |
|---|---|---|
| 0 | .315 | .300 |
| ½ | .290 | .290 |
| 1 | .280 | .275 |
| 1½ | .270 | .275 |
| 2 | .285 | .275 |
| 3 | .325 | .285 |
| 4 | .420 | .510 |
| 5 | .670 | .700 |
| 6 | .750 | .800 |
| 7½ | .860 | .94 |
| 8½ | .900 | - |

actinomycin D added to the resuscitation medium for S. aureus decreased the rate of synthesis and total amount of RNA produced. They interpreted this observation as indication that RNA synthesis was involved in the resuscitation of salt tolerance in heat damaged cells of S. aureus.

(ii) Ampicillin and Vancomycin

Both E. coli SA211 and SA603 resuscitated normally in the presence of ampicillin (2 ug/ml) and vancomycin (100 ug/ml) (compare data in Figure 4 and 5 to controls in Figure 3). For both strains, the presence of antibiotics in the resuscitation medium inhibited growth after the lag period. In the presence of vancomycin, resuscitation occurred during the first 3-4 hr. incubation at 37°C. Thereafter, the counts on both NA and VRBA decreased at an equivalent rate, indicating death without differential injury. In the experiments with vancomycin, the optical density data supported the fact that no growth occurred in the presence of vancomycin.

Both ampicillin and vancomycin interfere with cell wall formation, yet they have different sites of action. Ampicillin is bactericidal, and more active against Gram-negative bacilli than other penicillins. Hence its selection for use in these studies. Strominger (1967) found that ampicillin inhibited the enzymes peptidoglycan synthetase and D-alanine carboxypeptidase, in a far lower concentration than was necessary for the inhibition of growth of E. coli.

Vancomycin blocked amino acid incorporation into the cell wall mucopeptide (Jordan, 1961). It was also reported that vancomycin might affect cell wall synthesis indirectly, and

that the cytoplasmic membrane might represent the primary site of action. Shockman and Lampen (1962) examined the growth of Strep. faecalis spheroplasts and reported that penicillin had no effect, but that vancomycin inhibited the growth at concentrations which also inhibited growth of intact cells. Yudkin (1963) reported that vancomycin caused the decreased incorporation of labelled compounds into the membrane. He noted that 75% inhibition of incorporation occurred after 3 hr. This might explain the initial resuscitation of these cells in the presence of vancomycin, and the subsequent decrease in numbers observed with extended incubation in vancomycin containing broth (Figures 4 and 5).

(iii) Chloramphenicol and Tetracycline

Both of these antibiotics inhibited resuscitation, but in different ways. The results for both strains with chloramphenicol were similar (Fig. 6 and 7). From these figures it is apparent that with chloramphenicol at 2.5 µg/ml, resuscitation for growth on NA occurred, as well as an initial resuscitation for growth on VRBA. This was not indicated by Roth (1960), and the inference from his data was that no resuscitation occurred for VRBA (see Fig. 8). However, his data showed an increase during the first hour of plating onto VRBA and subsequent fluctuations in counts. After the initial resuscitation for VRBA, which occurred within 1 hr (SA603) and 1½ hr (SA211), no further resuscitation for VRBA occurred.

With the addition of tetracycline, 2.0 µg/ml, to the resuscitation broth for E. coli SA211, there was no initial

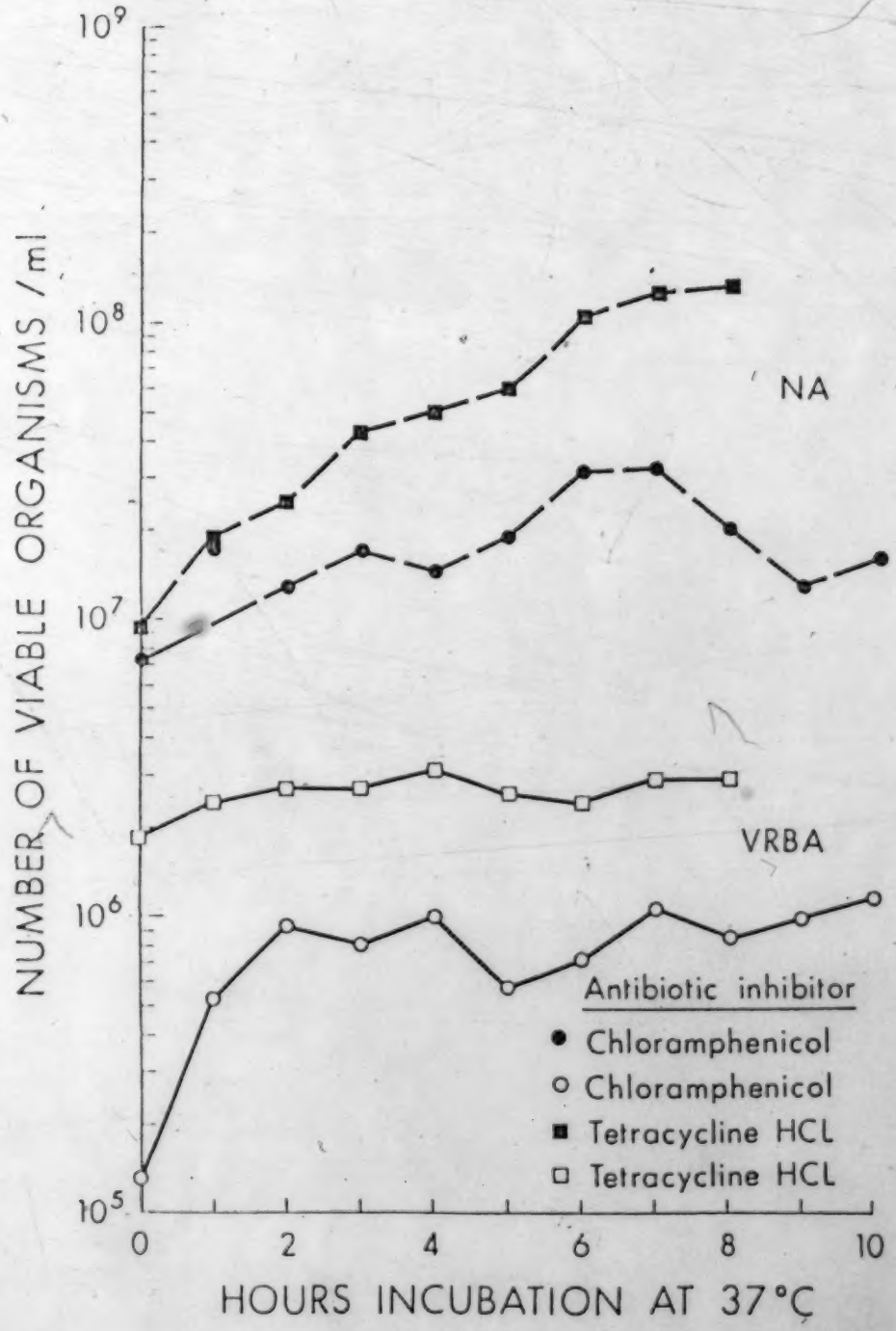


Figure 6. Resuscitation curves for E. coli SA211 in tryptic soy broth in the presence of minimum inhibitory concentration of chloramphenicol and tetracycline HCl.

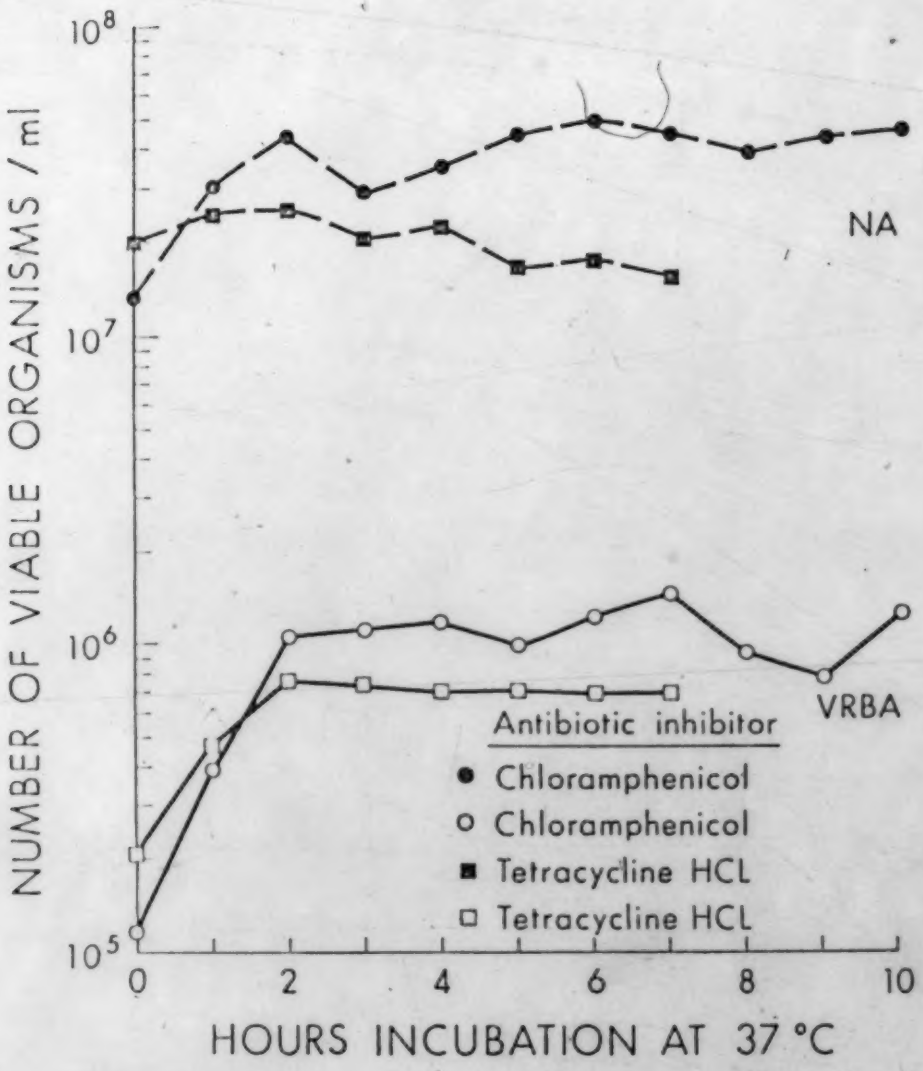


Figure 7. Resuscitation curves for E. coli SA603 in tryptic soy broth in the presence of minimum inhibitory concentration of chloramphenicol and tetracycline HCl.

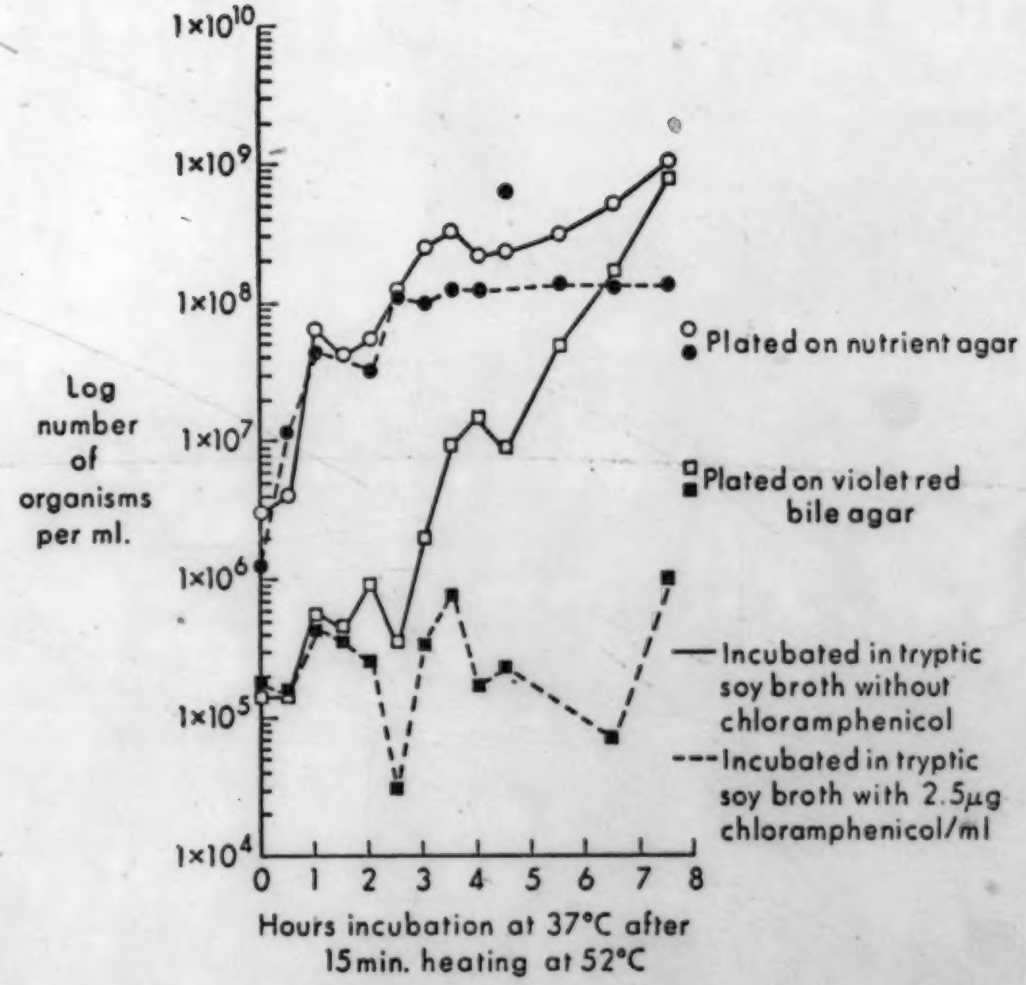
1×10^10
1×10^9
1×10^8
1×10^7
1×10^6
1×10^5
1×10^4
Log number of organisms per ml.
0 1 2 3 4 5 6 7 8
Hours incubation at 37°C after 15 min. heating at 52°C
Plated on nutrient agar
Plated on violet red bile agar
Incubated in tryptic soy broth without chloramphenicol
Incubated in tryptic soy broth with 2.5μg chloramphenicol/ml

Figure 8. Data presented by Roth (1969) for the resuscitation of heat treated E. coli SA211 with and without chloramphenicol.

resuscitation for VRBA. However, complete resuscitation occurred for NA (see Figures 6 and 7). The data for E. coli SA603 were less definite. In an initial experiment, SA603 gave the data shown in Fig. 7. Initial resuscitation occurred for VRBA, and the resuscitation process slowed after two hours. At the same time, the count on NA was decreasing.

Hahn and Wisseman (1951) discovered that chloramphenicol inhibited the induced synthesis of enzymes, and concluded that this effect indicated the inhibition of protein synthesis as the mode of action of this antibiotic. Direct proof of general inhibition of bacterial protein synthesis by chloramphenicol was obtained through biochemical analysis on E. coli (Wisseman et al., 1953, 1954), and for S. aureus (Gale and Folkes, 1953). Chloramphenicol blocks protein synthesis while allowing further synthesis of nucleic acids (Gale and Folkes, 1953). The site of inhibition has been shown to involve the transfer of amino acids from the transfer component of RNA to the peptide bond forming site in the ribosomes (Gale, 1963, and Davis and Feingold, 1962).

The tetracyclines are active chelating compounds, forming firm unions with divalent and trivalent cations, and may interfere with enzymes that require such cations as cofactors (Eagel and Saz, 1955). Gale and Folkes (1953) found that the tetracyclines inhibited protein synthesis, and had a similar action to chloramphenicol. It appeared in this study that chloramphenicol and tetracycline stopped the resuscitation of

the E. coli strains, but at different stages.

The difference in the mode of action of these antibiotics might account for the difference in the point at which resuscitation for VRBA was inhibited. From this data, it was apparent that resuscitation in E. coli involved the synthesis of protein, compared to the observation by Stiles and Witter (1965), that resuscitation of S. aureus occurred in the presence of chloramphenicol. Similarly, the data indicated that resuscitation of E. coli did not involve RNA synthesis. The synthesis of RNA was suggested by Iandolo and Ordal (1966) as a requirement for the resuscitation of S. aureus, after their observation that actinomycin D inhibited resuscitation.

Effect of nutrients on the resuscitation of E. coli

There have been many reports of better growth of both damaged and undamaged bacteria on enriched media (Dabbah et al., 1969; Harris, 1963; Heinmets et al., 1954; Moss and Speck, 1966; Mossel and Ratto, 1970; Nelson, 1943 and 1944; and Postgate, 1963). Response after damage was shown to depend substantially on the nutrient capacity of the medium. These studies were directed primarily towards the numbers of cells growing on culture media.

The following experiments were designed to investigate the effect of the nutrient capacity of the medium for resuscitation of heat treated E. coli.

Heat treated (55$^{o}$C for 15 min) E. coli SA603 were washed in distilled water and inoculated into tryptic soy broth (TSB)

and 0.1% peptone to resuscitate. Resuscitation in TSB was more rapid and more complete than in peptone water. Furthermore, the lag phase for the heat treated cells in TSB was two to three hours, whereas the lag phase for the same cells in peptone water was greater than 6 hr. From these studies, it was apparent that the richer medium supported greater resuscitation. Data indicated that with increasing concentration of peptone in the resuscitation medium, the level of resuscitation increased.

After observing that resuscitation occurred in the presence of 0.1% peptone, an experiment to determine whether amino acids or a simple nitrogen source would support resuscitation was carried out. The data are given in Table 5. The amino acids were mixed using 0.1% of each of the 20 amino acids reported to be present in peptone by Habeeb and Shotton (1955). Ammonium chloride and ammonium sulphate were selected as simple nitrogen sources. The amino acid mixture not only failed to support resuscitation, but also caused the rapid, continuing death of the heat treated cells, evidenced by decreasing counts on both NA and VRBA. Ammonium chloride gave a similar effect. However, ammonium sulphate did not cause continuing death of the heat treated cells. In fact, the data suggested the possibility that resuscitation might be supported. The effect observed for this simple nitrogen source warrants further study.

The emphasis of the following experiments is on the difference in the effect of peptone, compared to its constituent amino acids, for supporting the resuscitation of heat injured

Table 5. Resuscitation and growth response of E. coli SA603 in different resuscitation media.

| | Resuscitation* | Growth* |
|---|---|---|
| Tryptic soy broth (control) | ++ | 2-3 hr |
| Peptone, 0.1% (w/v) | + | 6 hr |
| Amino acid mixture | - | Death |
| $NH_4Cl$, 0.5% (w/v) | - | Death |
| $(NH_4)_2SO_4$, 0.1% (w/v) | - | - |

*Indicated by change in numbers of viable organisms on VRBA and NA.

cells. The effect of selected amino acids on the resuscitation of heat injured cells was studied. Selected amino acids were used at 0.1% (w/v) to make resuscitation media. The results are shown in Table 6. The results indicated that the individual amino acids had a deleterious effect on the injured cells. Both D,L-alanine and cysteine caused rapid death of the cells on both NA and VRBA; D,L-glutamic acid and D,L-lysine caused increased injury to the cells plated onto VRBA. Distilled water also showed the latter effect, however, saline showed an initial resuscitation for 2 hr., thereafter the resuscitation rate decreased and increased injury occurred.

Iandolo and Ordal (1966) indicated that phosphate was necessary for the resuscitation of heat injured S. aureus. Phosphate buffer, 0.1M, pH7.0 was used as a resuscitation medium, and by itself, did not support resuscitation. Added to peptone, it did not enhance the resuscitation rate. Furthermore, Stiles and Witter (1965) reported that an energy source, such as glucose or galactose in phosphate buffer was all that was required for the resuscitation of S. aureus at 37°C. An energy source, such as glucose (0.1%), added to phosphate buffer did not support the resuscitation of E. coli. However, the addition of glucose (0.1%) to peptone (0.1%) enhanced the resuscitation of the E. coli compared to peptone water alone. This confirmed that the nutrient capacity of the resuscitation medium had a marked effect on the resuscitation of the cells from heat damage. Unlike S.aureus, resuscitation of E. coli has not been

Table 6. Resuscitation and growth response of *E. coli* SA603 in selected amino acids and other potential resuscitation media

| | Resuscitation | Growth |
|---|---|---|
| Tryptic soy broth (control) | ++ | 2-3 hr |
| Peptone, 0.1% (w/v) | + | 6 hr |
| D,L-alanine, 0.1% (w/v) | - | Death |
| D,L-glutamic acid, 0.1% (w/v) | - | injury* |
| D,L-lysine, 0.1% (w/v) | - | injury* |
| Cysteine, 0.1% (w/v) | - | Death |
| Saline, 0.85% (w/v) | ± | - |
| Distilled water | - | injury* |
| Phosphate buffer, 0.1M, pH 7.0 | - | - |
| Phosphate buffer+glucose 0.1% | - | - |
| Peptone, 0.1% + glucose 0.1% | ++ | 6 hr |

* increased injury on VRBA, as opposed to death where counts were decreasing on both NA and VRBA.

demonstrated in a medium that does not support the normal growth of the cells. However, both ammonium sulphate and saline solutions used as resuscitation media indicated a slight resuscitation response without growth. Further study in this direction might also be justified.

The final studies in this section were carried out to observe the effect of peptone on resuscitation. The typical analysis for the Bacto-peptone used in these studies was obtained from Difco (Table 7). Habeeb and Shotton (1955) reported the amino acid composition of peptone using paper chromatography, and found that 20 identifiable amino acids and one unidentifiable "spot" were present. Difco cited only 13 amino acids and 5 vitamins. Hence the following study was carried out using peptone, 0.1%; a synthetic "peptone" 0.1% reconstituted according to Difco's typical analysis of peptone; and vitamin-free peptone, 0.1% (in which the vitamins were removed by dissolving the peptone in distilled water, adjusting to pH 8.0 to 8.5, using 30% NaOH. This mixture was boiled for 2 to 3 seconds, cooled and adjusted to pH 3.5 with concentrated HCl, stirred with 2 g activated charcoal on two occasions for 30 min, readjusted to pH 7.4 with NaOH and sterilized, according to Harries (1968). The results indicated that the factor(s) influencing resuscitation of the injured cells was not the vitamins. The cells failed to resuscitate on the synthetic "peptone", however they did resuscitate in vitamin-free peptone.

It would appear that the factor(s) supporting resuscitation of heat damaged E. coli in peptone water is in the peptide fraction. Moss and Speck (1966) reported that E. coli

Table 7. The typical analysis of Bacto-Peptone.*

| | |
|---|---|
| Ash | 4.0% |
| Total Nitrogen | 15.5% |
| Amino Nitrogen | 3.0% |

Amino Acids (in percentages)

| | |
|---|---|
| Arginine | 8.0% |
| Aspartic Acid | 6.0% |
| Glutamic Acid | 11.0% |
| Glycine | 23.0% |
| Histidine | 1.0% |
| Isoleucine | 2.0% |
| Leucine | 3.5% |
| Lysine | 4.5% |
| Methionine | 1.0% |
| Phenylalanine | 2.5% |
| Threonine | 1.5% |
| Tyrosine | 1.0% |
| Valine | 3.0% |

Vitamin Factors (micrograms per gram)

| | |
|---|---|
| Pyridoxine | 2.5 |
| Biotin | 0.3 |
| Nicotinic Acid | 35.0 |
| Riboflavin | 4.0 |

* Information from Difco, 1971
Difco Laboratories, Inc.,
Detroit, Michigan, U.S.A.

injured as a result of freezing, resuscitated when trypticase was added to the resuscitation medium, but that hydrolysis of the trypticase destroyed the ability of cells to resuscitate. This is probably a similar phenomenon to that being observed with heat damaged E. coli resuscitating in peptone water. Unlike S. aureus, a medium that supports resuscitation, without supporting the growth of the cells, has not been found for E. coli. However, it would appear that through extended study, possibly with saline, inorganic nitrogen and an energy source, some combination will be found that will provide this desirable, differential resuscitation medium.

## CONCLUSIONS

The data confirmed previous observations that media for the selective growth and differential enumeration of E. coli, do not support 100 percent growth of the potentially viable cells present in the culture. It is assumed that the counts obtained on the nonselective medium represent 100 percent of the potentially viable cells. As the counts on violet red bile agar were only 25 - 33 percent of the counts on nutrient agar, it may be concluded that selective media must be used with caution in estimating E. coli densities.

The coliform count is used as an indicator of hygienic standards in the food industry, and as an indicator of safety and hygiene by public health authorities. The failure of the selective media to support the growth of a fraction of the viable E. coli cells could lead to erroneous results. The importance of this phenomenon becomes more apparent in heat treated cells, when as many as 99 to 99.9% of the potentially viable cells fail to grow on the selective medium after relatively mild heat treatment. Such a deficiency in the selective medium could seriously hamper quality assurance or safety for the consumer. Either improved selective media, that will support the growth of injured cells, or resuscitation procedures that will allow injured cells to resuscitate, before growth, must be developed.

The necessity for re-appraisal of selective media for all organisms monitored by the food industry is of increasing

importance. With the increase in the number of convenience foods and the advent of "ready" foods, more of the foods available to the consumer are being processed in a way that causes the different forms of bacterial injury. Short time heating and low temperature storage are becoming the norm in food handling, especially for institutional use.

To develop adequate growth media or resuscitation procedures, more information on bacterial injury, and the mechanisms and conditions for resuscitation are necessary. Antibiotics were used to determine the resuscitation changes occurring in E. coli. The incorporation of actinomycin D allowed resuscitation to proceed. Hence it was concluded that RNA synthesis was not involved in the resuscitation of E. coli. Similarly, ampicillin and vancomycin failed to inhibit resuscitation while inhibiting growth. It was concluded that cell wall synthesis was not involved in heat injury.

The effects of ampicillin and vancomycin differ in that both allowed resuscitation to occur, but after 3 to 5 hr resuscitation in the presence of vancomycin, the cells started to die. The counts on both nutrient agar and violet red bile agar decreased at an equivalent rate, indicating the somewhat unusual phenomenon of death without differential injury. It was concluded that the death of the cells is probably due to the bactericidal action of vancomycin, however the observation of equivalent death rates on a selective and nonselective medium might prove to be useful in future studies of bacterial injury.

Chloramphenicol and tetracycline inhibit resuscitation. From the fact that both of these antibiotics, with their differing modes of action inhibit resuscitation, it was concluded that protein synthesis, as opposed to renaturation of the proteins was involved. This observation for E. coli clearly delineated the mechanism of resuscitation for this organism from that reported for S. aureus. The involvement of protein synthesis in E. coli, compared to RNA synthesis in S. aureus and Strep. faecalis, might represent a further difference between Gram positive and Gram negative.

The resuscitation studies were carried out in tryptic soy broth. This medium supported both resuscitation and growth. Many workers are recommending pre-enrichment of injured cells in this or similar nutrient broth cultures. Such procedures are only reliable as long as the post-heating lag or latency persists. Hence it would be desirable to find a resuscitation medium that does not support bacterial growth. From these data, it was concluded that removal of nutrients from the resuscitation medium increased the period of latency and decreased the rate of resuscitation. It was further concluded that the factor(s) supporting resuscitation in peptone was to be found in the peptide fraction. It was clearly demonstrated that single amino acid or amino acids mixtures have a deleterious effect on the resuscitating cells.

Future studies should be directed toward the development of a resuscitation medium that does not support growth. Such

a medium would be desirable for use in routine laboratories, where the use of growth supporting media for resuscitation might lead to errors or require excessive precautions. Although the addition of a protein synthesis inhibitor to tryptic soy broth might suffice, the results in this study indicate that a simpler alternative might be found.

The data suggest the need for further experimentation in the area of cell damage and reliability of selective cell counts on foods.

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